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Mira Mawardi

Main Center for Freshwater Aquaculture (MCFA), Ministry of Marine Affairs and Fisheries (MMAF). Jl. Selabintana No. 37, Sukabumi, West Java, Indonesia

Kei Yuasa

National Research Institute of Aquaculture, Fisheries and Education Agency (FRA), Japan

Ciptoroso

Main Center for Freshwater Aquaculture (MCFA), Ministry of Marine Affairs and Fisheries (MMAF). Jl. Selabintana No. 37, Sukabumi, West Java, Indonesia

Ayi Santika

Main Center for Freshwater Aquaculture (MCFA), Ministry of Marine Affairs and Fisheries (MMAF). Jl. Selabintana No. 37, Sukabumi, West Java, Indonesia

Zakki Zainun

Main Center for Freshwater Aquaculture (MCFA), Ministry of Marine Affairs and Fisheries (MMAF). Jl. Selabintana No. 37, Sukabumi, West Java, Indonesia

Correspondence

Mira Mawardi

Main Center for Freshwater Aquaculture (MCFA), Ministry of Marine Affairs and Fisheries (MMAF). Jl. Selabintana No. 37, Sukabumi, West Java, Indonesia

Evaluation of detection of koi herpesvirus disease by conventional polymerase chain reaction (PCR)

Mira Mawardi, Kei Yuasa, Ciptoroso, Ayi Santika and Zakki Zainun

Abstract

The method of detection of koi herpesvirus disease continues to be developed to obtain a high level of sensitivity and specificity. One common test method uses conventional PCR. This method resulting not only relatively inexpensive cost analysis but also rapid analysis. This experiment is using four types of commercial kits, the primers design of TK and Sph as well as some annealing temperatures. The sample used was the result of DNA extraction from koi gills, positive control of the collection of KHV 0301 FRA, positive control KHV with dilution concentration of 10000; 1000; 100; 10 and 1, to regulate the presence of contamination on amplification process, a negative control must be included, ddH₂O. The annealing temperatures used are 52 °C; 55 °C; 56 °C; 60 °C and 63 °C. This experiment showed different results of KHV detection. The sensitivity and specificity of the results are different for each kit, primers pairs and annealing temperature. Using a one-degree difference in annealing temperature will affect the detection of KHV although using the same kind of kits and primer pairs. The user is obliged to do optimization method regarding the kits usage, primers pairs and various temperatures to obtain a diagnostic method that has high level sensitivity and specificity detection. The sensitivity detection of KHV virus in this experiment could detect up to 10 copies. The user or analyst have to know and understand about all the information in the manual kits.

Keywords: Koi herpesvirus, primer, conventional PCR, annealing temperature, sensitivity, specificity

Introduction

Koi Herpesvirus Disease (KHVD) which is also known as viral gill necrosis or carp interstitial nephritis caused by Cyprinid herpesvirus 3 (CyHV-3) is an infection of a virus that generally infected the common carp and koi. This disease has occurred in several countries and has been reported in more than 30 countries [23, 33, 40, 27, 21, 3, 35, 12, 29, 26, 39, 16, 10, 45, 1, 18, 41, 42]. Koi herpesvirus infection can cause mortality of fish between 80-100% [1]. This disease is called a lethal disease in koi and common carp [35, 11, 29, 39].

The characteristics of clinical signs of KHV-infected on fish are skin lesions to the muscles, peeling or skinless epithelium, gill necrosis, deposit of thick mucus under the gill, mucus eroded so that the body of fish becomes rough [5, 1, 18, 42]. Fish's skin is as the gateway to KHV infection in fish [11]. So that fish are very susceptiblely infected by secondary pathogens such as bacteria, parasites or fungi. These infections can cause further death in fish populations [26, 1]. KHV infection occurs when the water temperature range is between 72 ° and 81 ° F (22° and 27 °C) [33, 30, 42, 45, 7, 27, 35] and at temperatures of 11-12 °C [2].

As seen in other species from other common carp, goldfish (*Carassius auratus*), grass carp, blue ideas (*Leuciscus idus*) and *Ancistrus* sp which have been infected by KHV and then detected using conventional PCR and viral isolation that shows negative result, these fishes are expressed as carrier of KHV [4, 26]. Whereas goldfish was positive infected with KHV using the real-time PCR detection method [38]. Goldfish x common carp hybrid are also very susceptible to KHV infection [22]. The fish that survive after an outbreak can be potentially become latent virus host [27, 35, 20].

The diagnostic identification of KHV can be done in several methods i.e., direct and indirect. The direct methods are a procedure that directly detects the presence of virus or "pieces" of virus (i.e., viral isolation in cell culture and identification of virus by the PCR method). Indirect methods are procedure which measure fish immune response by analyzing antibody levels [23, 33, 17, 24, 30]. The KHV diagnostic method using conventional PCR is commonly used [14].

The Koi herpesvirus is a long chain DNA molecule or head-to-tail concatemer, can replicate and as an intermediate host [25]. Therefore, we need a method that has a high level of sensitivity and specificity. Whereas conventional PCR sensitivity is considered lower than real-time PCR which can quantitatively detect KHV, like taqman's quantitative PCR. With 5 and 10 KHV DNA genomic equivalent can be detected by real-time PCR [15, 4, 20], nested PCR. Whereas there is not much information about the sensitivity and specificity of the KHV method detection using conventional PCR.

Material dan Method

Sample preparation

Samples were taken from koi fish farm in cages in Cirata lake, Cianjur, West Java, Indonesia in February 2016. Tissue samples were collected from gill filaments and preserved on 80% ethanol. The sample's codes are set as 1, 2, 3 and 20. Samples are then stored in the refrigerator in MCFA laboratory, Sukabumi. The samples were transferred to the new fixative solution (80% alcohol) then taken to FRA, Japan. During the trip the samples were kept on the room temperature. After arriving at the FRA laboratory, the samples were stored in the refrigerator until they were analyzed. The samples were extracted using an automatic extraction machine (Promega). The quality and concentration of DNA samples were calculated using a spectrophotometer (NanoVue, GE Healthcare). Testing the sample's codes 1, 2, 3

and 20 is also done using the real-time PCR method (Roche).

Positive control preparation

Positive KHV samples were taken from the results of cell cultures using infected CCB by KHV 0301 collected from FRA Japan. The viral supernatant was activated by heating the temperature of 99 °C for 5 minutes using a drying incubator then centrifuged at 3000xg for 5 minutes. The viral supernatant is taken as a positive control.

Preparation positive control of the KHV dilution. Preparation of the KHV with a concentration of 10⁵ copies results from cell culture. Preparation of fish genome as dilution solution is made from the results of negative KHV koi gill extraction. Preparation the dilutions of KHV with concentration from 10000; 1000; 100; 10 and 1 are diluted with a solution of fish genome.

Preparation of mastermix

This experiment is using some commercial kits that are (a) Ampli Taq Gold Applied Biosystem (ABI), (b) Takara Ex Taq Hot Star Takara, (c) Taq DNA Polymerase Qiagen and Platinum® and (d) Taq DNA Polymerase Invitrogen. Preparation of mastermix from each commercial kit is shown in table 1 below. This experiment is using two primer pairs that were TK and Gray Sph primers/Yuasa modification (Table 2) which are recommended by the World Organization for Animal Health [32].

Table 1: Preparation of master mix

Chemicals mastermix	X10 Buffer (µl)	dNTP (µl)	100 µm primer stock (µl)	Taq (µl)	MgCl ₂ (µl)	Distillation water (µl)
AmpliTaq Gold Applied Biosystem (ABI)	2	1.6	0.2	0.125	1.6	13.275
Takara Ex Taq Hot Star Version Takara	2	1.6	0.2	0.1	-	14.9
Taq DNA Polymerase, Qiagen	2	1.6	0.2	0.1	-	14.9
Platinum® Taq DNA Polymerase, Invitrogen	2	1.6	0.2	0.08	0.6	14.32

Table 2: Primer pairs for the detection of KHV

	Bercovier TK primers	Gray Sph-Yuasa modification
Primer sequences	5'-GGG-TTA-CCT-GTA-CGA-G-3' 5'-CAC-CCA-GTA-GAT-TAT-GC-3'	5'-GAC-ACC-ACA-TCT-GCA-AGG-AG-3' 5'-GAC-ACA-TGT-TAC-AAT-GGT-GGC-3'
Amplicon size	409 bp	292 bp
DNA sample	1 µl	1 µl
PCR reaction volume	20 µl	20 µl
Program	94 °C 5 min (1 cycle) 95 °C 1 min (40 cycles) 52 °C* 1 min 72 °C 1 min 72 °C 7 min (1 cycle)	94 °C 3 min (1 cycle) 94 °C 30 sec (40 cycles) 63 °C* 30 sec 72 °C 30 sec 72 °C 7 min (1 cycle)

*temperature annealing 52 °C; 55 °C; 56 °C; 60 °C and 63 °C.

Electrophoresis preparation.

The PCR product that has been added with 0.05% 10x loading dye is then spinned so that the sample material is on the bottom of the tube. Each 6 µl sample was then put into each of well of 2% agarose which had been given 1% etidium bromide. Using 100 bp ladder marker (promega) as a measurement of amplicon PCR products. Electrophoresis (Mupid) was carried out for 5 minutes and 135 volts and observations were carried out under UV lights.

Results

The following is a profile of KHV detection electrophoresis results. Code N is negative control (ddH₂O); P is a positive control. Code 1, 2, 3 and 20 are samples of gill koi tissue. Code -1; -2; -3; -4 and -5 are positive controls of fish which was infected by KHV with 10000; 1000; 100; 10 and 1 of dilutions of DNA concentrations.

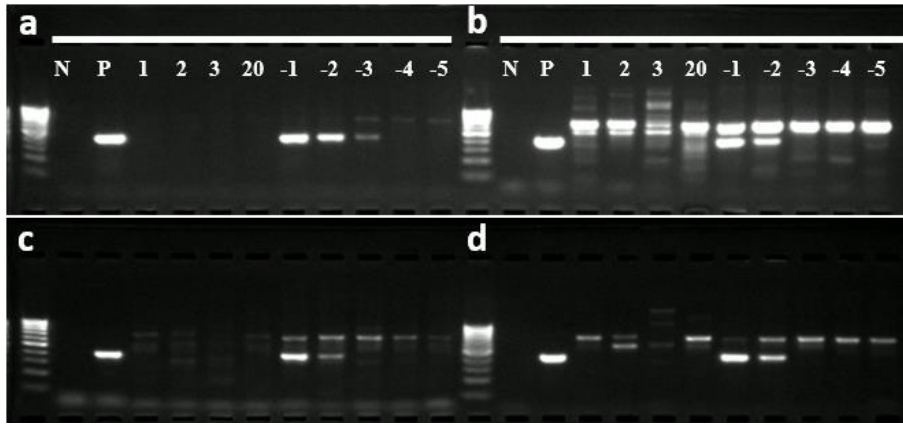


Fig 1: The amplification results used primer TK with annealing temperature at 52 °C

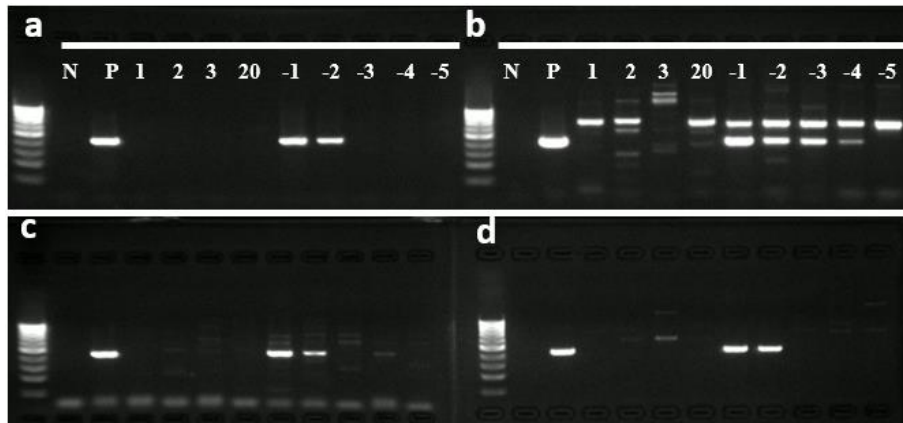


Fig 2: The amplification results used primer TK with annealing temperature at 55 °C

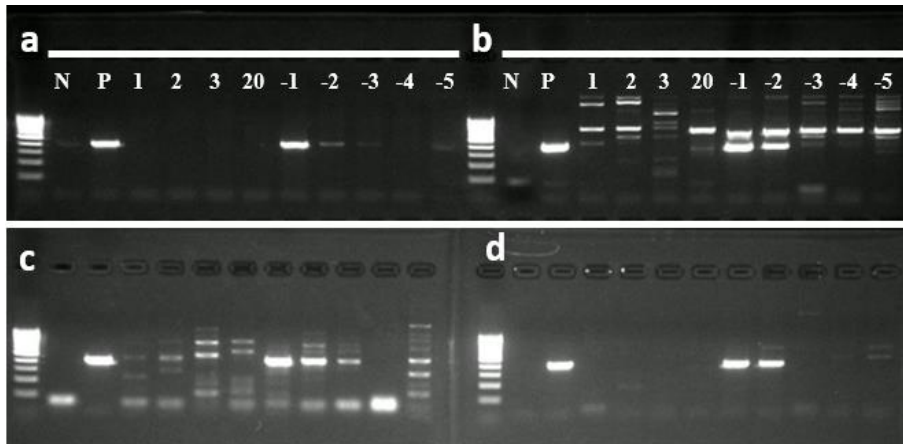


Fig 3: The amplification results used primer TK with annealing temperature at 56 °C.

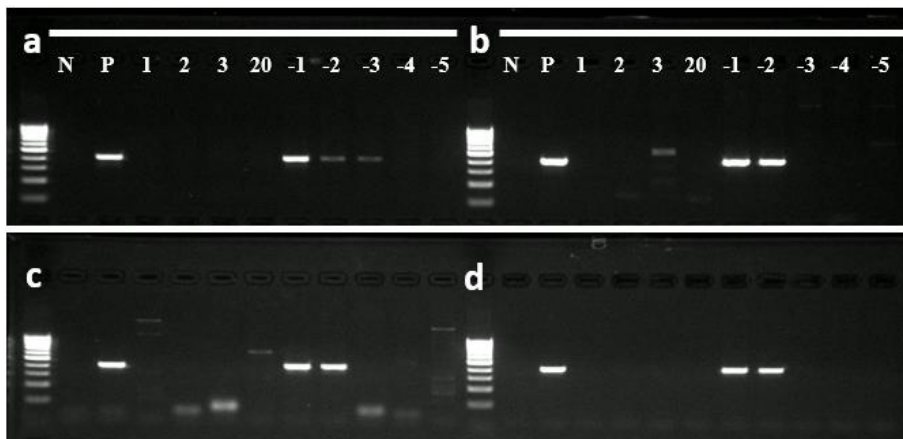


Fig 4: The amplification results used primer TK with annealing temperature at 60 °C

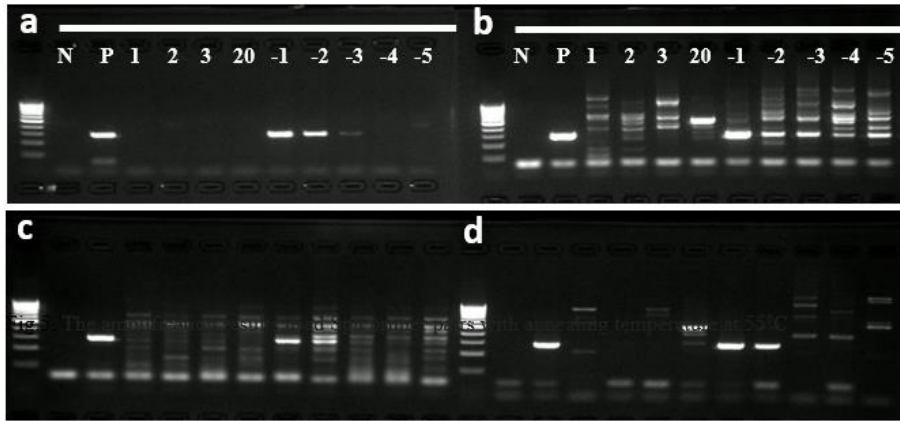


Fig 5: The amplification results used Sph primer pairs with annealing temperature at 55 °C

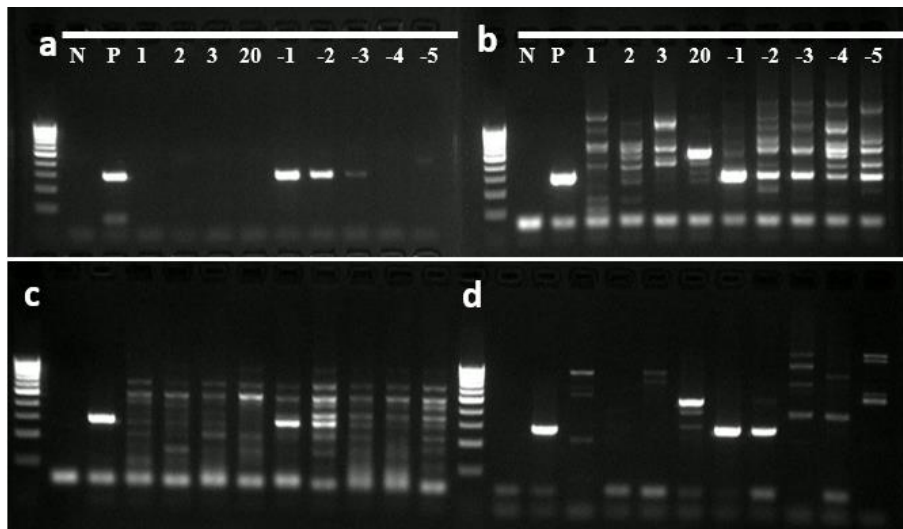


Fig 6: The amplification results used Sph primer pairs with annealing temperature at 63 °C

The overall results of the experiment showed that the KHV DNA band as a positive control (P) was very clear. The positive control with 10000 and 1000 concentration dilutions (codes -1 and -2) are also very clear in all results (Fig. 1; 2; 3; 4; 5 and 6). The positive control with 100 concentrations was almost all clear although not thick (Fig. 1.a; 3.a; 3.b; 3.c; 4.a and 6.a). While at a concentration of 10 and sample code 20, there was a thin band (Fig. 2.b). Based on the result, we can conclude that the same method will not always get the same result using different kits with the same primer pairs. As shown in Fig. 1 and Fig. 2, Each kit showed different results although using one different factor of method i.e., annealing temperature. It is highly possible that each kit has different optimum method to detect KHV with high sensitivity and specificity.

The results of sample codes 1, 2 and 3 using the real-time PCR are negative. These results are in accordance with the results of detection in this test. While code 20 samples were 15.4 copies virus with confirmation test using real-time PCR. If you see the results of some conventional PCR amplification with sample code 20, it shows negative. But there are also some results that show a positive band while the band look thin (Fig.2.b). At the time of sampling (code 1; 2; 3; and 20) there was no infection or outbreak of KHV disease in the area. There are also no clinical signs of KHV infection. The results showed level of sensitivity can detect up to 10 concentration of copies virus with the method. While for the other results (Fig.2.a; Fig.2.c and Fig.2.d), the method is not yet suitable to get a high level of sensitivity results. This shows high

opportunity that by using different kits have also different methods, such as primer concentration, temperature annealing and others.

The success of KHV diagnostic using several methods, kits and primer can reciprocally affect the result of sensitivity and specificity level. The presence of extra band or dimer primer is one of the correction factors for the amplification of the specificity results. The results of some tests shows many extra bands (other bands besides target bands). The existence of extra bands can be seen above and or below of band target. Band extras are also seen from the results of electrophoresis using TK and Sph primer pairs from several different use of annealing temperature (Fig. 1.a; 1.b; 1.c; 3.b; 3.c; 5.b; 5.c; 6.b; 6.c and 6.d). Although using the same kit with different annealing temperatures, it can also affect the presence of extra bands and dimer primers on amplification results.

Discussion

One of the spreading koi herpesvirus disease is through fish trade, including fish trade between countries. This disease is characterized by very rapid virus replication and spreading through fish organs which causes high fish mortality (starting on days 6 to 10 after infection) [34, 43, 21, 35, 38, 29, 36, 45, 9, 28, 31, 42, 37]. Several methods have been done to prevent transmission of KHV diseases including using chemicals and implementing biosecurity systems. Cases of KHV infection also occur in public waters in several countries. The high level of losses that have occurred in several countries have made The World Organization for Animal Health determined that KHV disease

is one of wary disease and included in the category of dangerous diseases. Therefore, the OIE establishes several detection methods for the disease.

The methods for detection of KHV have been developed to obtain high sensitivity, specificity and repeatability. These are conventional PCR method, Nested PCR assays [13], quantitative real-time PCR [15] and loop-mediated isothermal amplification (LAMP) [19]. The conventional PCR method has a high sensitivity level for detecting KHV in infected fish [14, 47, 4, 26, 36, 46]. The diagnosis of KHVD in fish is also effective using the virus isolation method with cell culture [32]. The brain of fish infected with KHV are the best samples used as KHV virus isolation [48]. However, these methods are not recommended to be used for fish that live after infection or are carriers [47, 6]. Detection methods using conventional PCR are rated fast and economical. But in fact the application of the method is not necessarily as easy as written in the testing manual. Based on this experiment, some factors affect the results. Therefore, it is very important to determine which level of sensitivity and specificity test method that will be used.

Conventional polymerase chain reaction (PCR) is a common method used for diagnostic KHV. Many factors affected the results, including personal skills, availability of equipment, the materials and consumables used. In accordance with that, one of the most important technical factors in testing is the determination of the test method relating to the use of kit, primer pairs, primer concentration, amplification cycle and the quality and quantity of sample DNA. As in this experiment, optimization of the method is done by using many kinds of kits, primer pairs and some annealing temperatures. These three factors reciprocally affect sensitivity and specificity of KHV detection results.

Based on this experiment, using four kinds of kits, the detection results were very different even though using the same primer, cycles amplification and samples. High sensitivity and specificity for detection of KHV are not necessarily obtained when using the same primer and method but different kit. So if a laboratory uses several kinds of kits, it will be necessary to have method optimization to obtain a high level of sensitivity and specificity detection. The kits used are generally commercially available. To get the kit, it is often constrained by the availability of these materials. Such as the kits that are not always ready stock, the time of import kits, and the short expiration date of kit. So that some laboratories use several kinds of kits. Every kits always includes a manual in its package. It is recommended that the user must know all kit usage information, such as the composition of the kits, the supporting material, concentration and the recommended amount for each reaction, how to use and other information. If the information contained in manual packaging is limited, user should search for information online on the product website. By following the recommended method in the kit manual, the chances of getting the method with great sensitivity and specificity will be even higher.

Using the same kit and primer with different methods obtaining different sensitivity and specificity result for KHV detection. As in Fig. 2.b, it can be seen that the sensitivity of the detection of positive control KHV dilution can be at 10 concentrations. Similarly, it can also be seen in the code sample 20 which has a concentration of 15.4 copies. Virus is also positively detected even though the band's brightness level is smear. Whereas the results in using different methods

show different level of sensitivity and specificity (Fig.1.b; Fig.3.b; Fig.4b; Fig.5.b and Fig.6.b). Similar results are also seen in other kits.

The high level of sensitivity and specificity method for KHV detection is not necessarily obtained by using the same primer and kit but different methods. Primer use is very important to be noted, including the concentration of the stock, the volume of each reaction and the final concentration in the reaction. Stock concentrations of primers pairs should be stored in low concentrations (10-20 μM). This is to avoid contamination in the primer stock solution so that we can make new primer stock solutions from the previous primary master sheet solution. The final concentration primer written on each manual kit is very diverse. Usually the recommended final concentration values are between 0.1-1 μM . The final concentration recommended by using Sph primer is 0.5 μM . Whereas for TK primer is 0.1 μM [32]. Some laboratories use final value of TK primer 0.25-1 μM and Sph primer 0.4-0.5 μM [44, 8] as final concentration. Based on various final concentration value stated on each manual kit, it is very important to perform method optimization referring to those listed in the manual kit used. As in this experiment, using the final concentration of 1 μM for some kinds of kits resulting different detection result on the sensitivity and specificity of KHV. Although the final concentration is still within recommended range, but from the results of this experiment, it can be seen that each kit do not match with the final value of the same concentration primer. Using the same concentration on primer pairs TK and Sph could get different detection results. As seen in this experiment, it was proven that by using the same kits, the same amplification cycle, the same final concentration primer, the same sample and processing time, but different primer pairs show very different results (Fig.2 and Fig.5). It can be said that using primer TK and Sph leads to different optimum final value of primer concentration.

Determination of cycle, time and temperature of amplification usually refers to the kits and the primer used. In general, the temperature, time, and number of amplification cycles listed in the manual kit are range in value. Whereas normally in primer's manual is stated suggested range of annealing temperature. Based on the results of the gel electrophoresis profile in figures 1 and 2, it can be seen that using only one-degree difference of annealing temperature could get different detection profile of KHV DNA on each kit and the same primer. The annealing temperature by TK primer is 52 $^{\circ}\text{C}$ and many laboratories use 55 $^{\circ}\text{C}$ while for Sph primer is 63 $^{\circ}\text{C}$ [32]. As in the results Fig. 2 and Fig. 3, the usage of each kit with different annealing temperatures results in different detection level. Similarly, Fig. 5 shows each kit also produces different levels of sensitivity and specificity. Based on this test, it can be seen that the determination of annealing temperature greatly effects sensitivity and specificity of KHV detection.

Conclusion

Analysis of koi herpesvirus disease using conventional PCR is one of the fastest detection methods. It is very important to optimize the method to determine the level of sensitivity and specificity of the results so that the method can be determined as a testing method in the laboratory. User must follow the instructions contained in each kit manual. Each kit, primers and cycles amplification used can effect to the results of the level of sensitivity and specificity detection. This experiment proves that the sensitivity of koi herpesvirus detection using

conventional PCR can detect up to 10 copies. This experiment could inform and develop the optimization of conventional PCR methods for future detection of KHV.

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